REMARKS

Claims 16, 17, 19-21 and 30-42 were pending in the application. Claim 16 has been amended. Accordingly, after the amendments presented herein have been entered, claims 16, 17, 19-21 and 30-42 will remain pending in the instant application. *No new matter has been added.*

Support for the amendments to claim 16 can be found throughout the specification and claims as originally filed.

Cancellation of and/or amendment to the claims should in no way be construed as an acquiescence to any of the Examiner's rejections. The cancellation of and/or amendments to the claims are being made solely to expedite prosecution of the above-identified application. Applicants reserve the option to further prosecute the same or similar claims in the instant or in another patent application.

Withdrawal of Rejections

Applicants gratefully acknowledge the withdrawal of the Examiner's rejection of claims 16-17,19-22, and 30-34 under 35 U.S.C. §112, first paragraph, and the rejection of claims 16-17, 19-22, and 30-34 under 35 U.S.C. §112, second paragraph.

Rejection of Claims 16-17, 19-21 and 30-42 Under 35 U.S.C. §112, First Paragraph

The Examiner has rejected claims 16-17,19-21, and 30-42 under 35 U.S.C. §112, first paragraph, because, according to the Examiner,

> [c]laims 16-17, 19-21, 30-42 are overly broad since insufficient guidance is provided as to which of the myriad of possible nucleic acid sequences serve as IgE promoters, and Applicant only discloses an IL-4 inducible epsilon promoter of SEQ ID NO: 1. Since the claims encompass variant nucleic acids, it would require undue experimentation to make and use the claimed invention. See In re Wands, 858 F.2d at 737, 8 USPQ2d at 1404.

> The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. Applicant is required to enable one of skill in the art to make and use the claimed invention, while the claims encompass polynucleotides that the specification only teaches one skilled in the art to test for functional variants. It would require undue experimentation for one of skill in the art to practice the claimed method, since the skilled artisan would have to first make the polynucleotide variants, but there is no functional limitation set forth for the polynucleotide variant.

Applicants respectfully submit that one of ordinary skill in the art would be able to make and use the claimed invention, including the IgE promoter used in the methods of the invention, using only routine experimentation.

As stated by the Examiner, in order for a claimed invention to be enabled, the standard is not whether or not experimentation is necessary to practice the claimed invention. Rather, the standard is whether or not the experimentation necessary to practice the claimed invention is undue (See *In re Wands*, 858 F.2d at 737). Thus, enablement is not precluded by the necessity for some experimentation, and a considerable amount of experimentation is permitted. *In re Wands*, supra. Applicants provide sufficient guidance such that one of ordinary skill in the art could practice the methods claimed in claim 16, without undue experimentation.

One of ordinary skill in the art, armed with the knowledge of one of the ordinarily skilled artisan and given the teachings and methods disclosed in Applicants' specification, would be able to practice the claimed methods using IL-4 inducible IgE promoters taught in Applicants' specification or identifying additional IL-4 inducible IgE promoters, using no more than routine experimentation.

Contrary to the Examiner's assertion, at the time the instant application was filed, it was conventional in the art for one of ordinary skill in the art to test IL-4 inducible IgE promoters for

function by, for example, assaying for induction of an IL-4 inducible IgE promoter in the presence of IL-4 or IL-13. Methods for testing for IL-4 inducible promoter function are also described in Applicants' specification. For example, as set forth in Applicants' specification "introduction of IL-4 causes the pronounced activation of a particular DNA binding protein that then binds to the IL-4 inducible promoter segment and induces transcription" (see page 8, lines 7-9). Moreover, Applicants' specification states that modulation of an IL-4 promoter may be measured by the presence or quantification of transcripts or of translation products, *e.g.,* in the presence of IL-4 or IL-13 (see, for example, page 8, lines 3-18).

Furthermore, the Examiner states that "the specification only teaches one skilled in the art to test for functional variants." Applicants respectfully submit that the claimed methods utilize only ***functional*** IgE promoters. Non-functional IgE promoters are not encompassed in the methods of the invention.

Moreover, at the time the instant application was filed, IL-4 inducible IgE promoters were very well-characterized and significant structural information regarding IL-4 inducible IgE promoters was known in the art. As evidence of the extensive characterization of these promoters, Applicants provide herewith copies of Delphin and Stavnezer (1995) *J. Exp. Med.* 181:181-192 (attached hereto as Appendix A) and Ezernieks, *et al.* (1996) *Eur J. Biochem.* 240(3):667-73 (attached hereto as Appendix B). For example, Delphin and Stavnezer describe IL-4 responsive regions within the germline epsilon promoter which are highly conserved and necessary for induction of the promoter by IL-4. Ezernieks, *et al.* describe a 51 base-pair fragment of the human IL-4 inducible promoter that confers upregulation of transcription in the presence of IL-4 or IL-13. Based on the knowledge available in the art at the time the application was filed, including the structural and functional characterization of IL-4 inducible

promoters, *i.e.,* the characterization of relevant conserved responsive regions contained within the promoters, one of ordinary skill in the art would have been able to make and use these promoters.

Therefore, it is Applicants' position that, given the guidance in Applicants' specification and the characterization of IL-4 inducible IgE promoters in the art at the time of filing, one of ordinary skill in the art would be able to make and use the functional IL-4 inducible IgE promoters used in the methods of the invention, including those having sequences other than SEQ ID NO:1, using no more than routine experimentation.

However, in an effort to expedite prosecution of the instant application, and in no way acquiescing to the Examiner's rejection, Applicants have amended claim 16 to refer to an IL-4 inducible promoter comprising the sequence set forth as SEQ ID NO:1. Accordingly, Applicants respectfully request withdrawal of the foregoing rejection.

Rejection of Claims 16-17, 19-21 and 30-42 Under 35 U.S.C. §112, First Paragraph

The Examiner has also rejected claims 16-17, 19-21, and 30-42 under 35 U.S.C. 112, first paragraph, "as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention, for reasons of record set forth in paper NO. 10, 9/26, 2002." In particular, the Examiner is of the opinion that

> [t]he claims are directed to methods of screening using a construct comprising an IgE promoter, a ε heavy chain and a reporter gene, while Applicant only discloses an IL-4 inducible epsilon promoter of SEQ ID NO: 1. The specification and claim do not indicate what distinguishing attributes shared by the members of the genus. The specification and claim

> do not place any limit on the number of substitutions, deletions, insertions and/or additions that may be made to the promoter. Thus, the scope of the claim includes numerous structural variants, and the genus is highly variant because a significant number of structural differences between genus members is permitted. The specification and claim do not provide any guidance as to what changes should be made. Structural features that could distinguish compounds in the genus from others in the nucleic acid class are missing from the disclosure. No common structural attributes identify the members of the genus. The general knowledge and level of skill in the art do not supplement the omitted description because specific, not general, guidance is what is needed. Since the disclosure fails to describe the common attributes or characteristics that identify members of the genus, and because the genus is highly variant, a nucleic acid with a sequence as set forth in SEQ ID NO: 1 is insufficient to describe the genus.

Applicants respectfully traverse the foregoing rejection. Applicants note that "[a] specification may, within the meaning of 35 U.S.C., § 112, first paragraph, contain a written description of a broadly written claimed invention ***without describing all species that claim encompasses***." *Utter v. Hiraga*, 845 F.2d 993, 6 USPQ2d 1709 (Fed. Cir. 1988).

Applicants have described a genus of IL-4 inducible IgE promoters based on their ability to be induced by IL-4 and IL-13. Applicants provide description of a sufficient variety of exemplary species to reflect the variation within the genus of IL-4 inducible IgE promoters used in the methods of the invention. Furthermore, at the time the instant application was filed, IL-4 inducible IgE promoters were well-characterized in the art, as evidenced by the Delphin and Stavnezer reference (Appendix A) and the Ezernieks, *et al.* reference (Appendix B). As set forth above, these references describe distinguishing attributes, including structural features, which are shared by the members of the genus of IL-4 inducible IgE promoters. For example, Delphin and Stavnezer describe IL-4 responsive regions within the germline epsilon promoter which are highly conserved and necessary for induction of the promoter by IL-4. Ezernieks, *et al.* describe a 51 base-pair fragment of the human IL-4 inducible promoter that confers upregulation of

transcription in the presence of IL-4 or IL-13. The sequence set forth in Figure 2A of the Delphin and Stavnezer is approximately 75% identical to the sequence set forth in SEQ ID NO:1 over nucleotides 395-564 of SEQ ID NO:1. Therefore, these sequences share a high degree of homology over the epsilon conserved sequence described in this reference.

Applicants' respectfully submit that promoters which are encompassed by the methods of the claims are limited to those functional IgE promoters which are capable of being induced by IL-4, as described in Applicants' specification, and as set forth above. Therefore, these promoters contain ***distinguishing functional characteristics***, *e.g.,* the ability to induce transcription in the presence of IL-4. An example of the sequence of an IL-4 inducible IgE promoter is set forth in Applicants' specification.

Furthermore, the identification or production of variant nucleic acid sequences was well-known in the art at the time the application was filed. Testing the variant promoters for the ability to be induced by IL-4 was also known to those of skill in the art and is described in Applicants' specification. Thus, based on the teachings in Applicant's specification, one of skill in the art would conclude that Applicants were in possession of the claimed invention at the time of filing.

The description contained in Applicants' specification coupled with the known correlation between function and structure is sufficient to satisfy the requirements of 35 USC §112 as set forth in the Written Description Guidelines (66 Fed. Reg at 1106) and by the court in *Enzo Biochem, Inc. v. Gen-Probe Inc.* (296 F.3d 1316 (Fed. Cir. 2002). However, in an effort to expedite prosecution of the instant Application, and in no way acquiescing to the Examiner's rejection, Applicants have amended claim 16 to refer to an IL-4 inducible promoter comprising

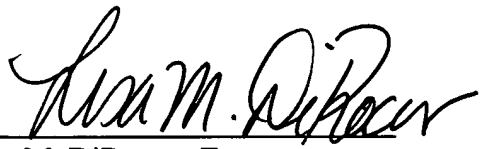
the sequence set forth as SEQ ID NO:1. Accordingly, Applicants respectfully request withdrawal of the foregoing rejection.

CONCLUSION

If a telephone conversation with Applicants' Attorney would expedite prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 227-7400.

Respectfully submitted,

LAHIVE & COCKFIELD, LLP

By: 

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Dated: **October 27, 2004**

APPENDIX A

Characterization of an Interleukin 4 (IL-4) Responsive Region in the Immunoglobulin Heavy Chain Germline ε Promoter: Regulation by NF-IL-4, a C/EBP Family Member and NF-κB/p50

By Sandra Delphin and Janet Stavnezer*

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Summary

A large body of data indicate that antibody class switching is directed by cytokines by inducing or repressing transcription from unrearranged, or germline, C_H genes. Interleukin 4 (IL-4) induces transcription of the germline Cε genes in activated B cells and subsequently, cells in this population will undergo switch recombination to immunoglobulin E. Furthermore, the data suggest that transcription of germline Cε genes is required for class switching. In this paper we define DNA elements required for induction of transcription of the germline Cε genes by IL-4. To do this, segments of DNA from the 5′ flank of the initiation sites for germline ε RNA were ligated to a luciferase reporter gene and transfected into two mouse B cell lines, one of which can be induced to switch to IgE. By analysis of a series of 5′ deletion constructs and linker-scanning mutations, we demonstrate that a 46-bp segment (residing at −126/−79 relative to the first RNA initiation site) contains an IL-4 responsive region. By electrophoretic mobility shift assays, we find that this segment binds three transcription factors: the recently described NF-IL4, one or more members of the C/EBP family of transcription factors, and NF-κB/p50. Mutation of any of the binding sites for these three factors abolishes or reduces IL-4 inducibility of the ε promoter. A 27-bp segment within this IL-4 response region containing binding sites for NF-IL4 and a C/EBP factor is sufficient to transfer IL-4 inducibility to a minimal c-fos promoter.

Antibody class switching is effected by a DNA recombination event that results in the substitution of one Ig heavy chain constant region (C_H) gene for another, thus changing the C_H region and thereby the effector function of the antibody, but keeping the antigen binding specificity unchanged. The specificity of class switching is regulated by cytokines that direct switching by inducing or repressing transcription from unrearranged or germline C_H genes before switch recombination to the same C_H gene (1–7). For example, a large body of data indicate that IL-4 induces transcription of the germline Cε gene in B cells activated by polyclonal activators, e.g., LPS, EBV infection or anti-CD40 antibody, and that subsequently cells in this population will undergo switch recombination to the Cε gene, thereby allowing expression of IgE (7–11). Furthermore, it has recently been shown that if a DNA segment which contains the promoter and first exon (I, or germline exon) of the germline transcript is deleted, switching does not occur on that chromosome (5, 6, 12). Although the sequences necessary and sufficient for switching have not been defined, these gene knockout experiments indicate that transcription of germline transcripts is probably necessary for switching. Therefore, it is important to understand how transcription of unrearranged C_H genes is regulated.

A map of the mouse germline ε DNA segment and transcript is shown (see Fig. 1 *A*). The DNA sequences which regulate transcription of the mouse and human germline ε RNAs are currently being studied. Initial characterizations of DNA sequences which regulate induction by IL-4 have been published (13–15). In this report we characterize an additional IL-4 responsive region (IL-4RR)[1] in the mouse germline ε promoter that overlaps with the previously characterized IL-4RR of the human germline ε promoter (14). This ε IL-4RR resides within a DNA sequence highly conserved between human and mouse and also has elements similar to those within an IL-4RR within the promoter for mouse germline γ1 transcripts (16, 17).

Materials and Methods

Cell Lines. Two mouse B lymphoma cell lines were used in this study: 22A10, a clone of the sIgM$^+$ I.29μ B cell lymphoma line (18, 19) and M12.4.1, an Ig-negative, class II$^+$, HGPRT-

[1] *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; IL-4RR, IL-4 response region; mt, mutant.

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deficient variant of M12.4 (20), received from Dr. Paul Rothman (Columbia University, New York).

Cell Culture. 22A10 cells were cultured as described for I.29μ cells (21). M12.4.1 cells were cultured at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), 50 μM 2-ME, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 200 U/ml penicillin, 200 mg/ml streptomycin, and 0.1 mg/ml kanamycin sulfate. Cells were treated with murine rIL-4 (a gift from Dr. Steven Gillis, Immunex Corp., Seattle, WA and the Sterling Research Group, Malvern, PA) at 1,000 U/ml, unless otherwise specified. In initial experiments, LPS (055:B5; Sigma Chemical Co., St. Louis, MO) dissolved in RPMI, was added at 25 μg/ml of culture.

Splenic B Cell Cultures. Splenic B cells were purified and cultured as described (22), except total B cells were used rather than only small B cells, and cells were cultured for 12 h with IL-4 at 1,000 U/ml and LPS at 25 μg/ml, as indicated.

Isolation of RNA and RNA Blot Analysis. Total cell RNA was prepared by the guanidinium isothiocyanate-CsCl method (23) and RNA blots were prepared and hybridized as described (21). Quantitation of hybridization was performed by densitometry on a Betascope 603 blot analyzer (Betagen, Waltham, MA). The Cϵ probe was a 2.1-kb BamHI/HindIII genomic DNA fragment (24). Hybridization to a GAPDH probe (25) or densitometry of 18S rRNA in photos of ethidium bromide–stained agarose gels was used to normalize the difference in loading.

RNase Protection Assay. To determine the initiation site of germline ϵ RNA, RNase protection assays were performed (26). Total cell RNA was prepared from 22A10 cells induced with an IL-4–containing supernatant from X63Ag8-653-IL-4 for 48 h (27). The X63 supernatant was titrated for germline ϵ RNA induction and used at an optimal dose. The RNase protection probe was transcribed from a 512-bp HincII/PstI genomic DNA fragment encompassing the Iϵ exon (28, 29) and cloned in Bluescript KS$^-$. The plasmid was linearized with HincII and transcribed in vitro with T7 polymerase. 100 μg of total cell RNA was incubated with 250,000 cpm of the labeled RNA probe in a 30-μl volume containing 80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, and 1 mM EDTA at 80°C for 10 min, and then hybridized overnight at the indicated temperatures. Unhybridized RNA was digested at room temperature for 90 min in 10 mM Tris, pH 7.4, 5 mM EDTA, pH 7, 300 mM NaCl, 2 μg/ml RNase T1 (Sigma Chemial Co.), and 40 μg/ml RNase A (Sigma Chemical Co.). The hybridized RNA was recovered and electrophoresed on an 8-M urea sequencing gel alongside a DNA sequencing ladder.

Sequences of Oligonucleotides. Lowercase letters are nucleotides added to create restriction enzyme sites and are not in the ϵ promoter sequence. ϵ promoter oligonucleotides are: A-1 (32 mer): 5′ gatccTGCCTTAGTCAACTTCCCAAGAACAGA 3′; A-2 (32 mer): 5′ gatcTCTGTTCTTGGGAAGTTGACTAAGGCAG 3′; Amt13-1 (31 mer): 5′ gatccTGCCCCTCGAGGTTCCCAAGAACAGA 3′; Amt13-2 (31 mer): 5′ gatcTCTGTTCTTGGGAACCTCGAGGGGCAG 3′; Amt20-1 (32 mer): 5′ gatccTGCCTTAGTCAACTTCCCCCTCGAGGA 3′; Amt20-2 (32 mer): 5′ gatcTCCTCGAGGGGGAAGTTGACTAAGGCAG 3′; B-1 (19 mer): 5′ gatccAAGGGAACTTCCAA 3′; B-2 (19 mer): 5′ gatcTTGGAAGTTCCCTTG 3′; Bmt27-1 (19 mer): 5′ gatccAAGGGCCTCGAGGA 3′; Bmt27-2 (19 mer): 5′ gatcTCCTCGAGGCCCTTG 3′; C-1 (16 mer): 5′ gatcCCCAAGAACAGA 3′; C-2 (16 mer): 5′ gatcTCTGTTCTTGGG 3′; D-1 (19 mer): 5′ gatccTTCCCAAGAACAGA 3′; D-2 (19 mer): 5′ gatcTCTGTTCTTGGGAAG 3′; E-1 (18 mer): 5′ gatcCCTTAGTCAACTTCA 3′; E-2 (18 mer): 5′ gatctGAAGTTGACTAAG 3′; 5′ϵ (−788/−733) (28 mer): 5′ttagatctGACCTGCAGCTGAGACAGAC 3′; and 3′ϵ (+33/+53) (28 mer): 5′ ttagatctTGTGCAGGCTCCCAGGCGTT 3′. Non ϵ promoter oligonucleotides are: NFκB1 (27 mer): 5′ CAACGGCAGGGGAATTCCCCTCTCCTT 3′; NKκB2 (27 mer): 5′ AAGGAGAGGGGAATTCCCCTGCCGTTG 3′; Luc (+79/+96) primer (18 mer): 5′GGCCCGGCGCCATTCTAT 3′ (30); Fos (−25/−8) primer (18 mer): 5′GGCGCCTCAGCTGGCGCC 3′ (31); and pXP2 polylinker primer (17 mer): 5′ AGATCCAAGCTTGTCGA 3′. Oligonucleotides were obtained from the University of Massachusetts Medical School nucleic acid facility, Operon Technologies Inc. (Alameda, CA), or DNA International (Woodlands, TX).

ϵ Promoter-Luciferase Plasmids. To create the 5′ deletion constructs of the germline ϵ promoter, a series of fragments containing various lengths of the promoter segment were ligated into the plasmid pXP2, which has a polylinker but not a promoter upstream of the luciferase (Luc) reporter gene (32). The 3′ border of these promoter fragments was created by PCR amplification of genomic ϵ DNA, using the primers, 3′ϵ (+33/+53 relative to the first start site) and 5′ ϵ (−788/−773). Nucleotide +53 is within the Iϵ exon and is located 5′ to the potential protein synthesis initiation codon at +65. The 5′ borders were created by restriction enzyme digestion at BamHI (1.8-kb promoter) or BglII (1.2-kb promoter), cloned into the BglII site of pXP2, or by digestion with BstXI (640-bp promoter), HincII (115-bp promoter), or AvrII (15-bp promoter), and cloned into pXP2 digested with SmaI and BglII. BstXI digestion followed by Bal31 digestion created additional smaller promoters that were cloned into pXP2 digested with SmaI and BglII. The nucleotide sequences of all plasmids having 640 bp or less of the promoter were verified by sequencing from the 5′ direction using a primer complementary to the polylinker of pXP2 (pXP2 primer) and also from the 3′ direction, using the Luc primer, extending as far as possible.

Linker-scanning Mutations. 8-bp Xho linker substitutions were introduced into the full-length promoter-luciferase reporter construct, −162Luc, by using Bal31 to generate a nested set of 5′ and 3′ deletion mutants, bounded by XhoI linker. The deletion end points were determined by nucleotide sequencing. 5′ and 3′ deletion clones with matching end points were chosen as best as possible to attempt to preserve the length of the wild-type promoter. Ligation of a BamHI (in polylinker)/XhoI fragment from a 3′ deletion clone into a 5′ deletion plasmid digested with BamHI and XhoI resulted in the regeneration of an approximately full-length promoter (in most cases) containing an 8-bp XhoI linker substitution. All constructs were sequenced across the sites of mutation using the pXP2 or Luc primers.

pFL Reporter Plasmids. Plasmid pFL contains the mouse c-Fos 71-bp promoter segment from pfosCAT (33) (given by M. Lenardo, National Institutes of Health, Bethesda, MD) ligated 5′ to the luciferase gene (34). Double-strand oligonucleotides containing BamHI sticky ends at the 5′ end and BglII sticky ends at the 3′ end were phosphorylated with γ-[^{32}P]ATP with T4 polynucleotide kinase at 1 U/μl (Boehringer Mannheim, Indianapolis, IN), 2% wt/vol PEG 8000, 75 ng/μl BSA, and 1 mM ATP at 37°C for 30 min. The phosphorylated oligonucleotides were multimerized and ligated into the BamHI site of plasmid pFL. Recombinant clones were screened by PCR amplification using the same ϵ oligonucleotides as primers individually in combination with a primer complementary to the c-Fos promoter. PCR products obtained using the top strand oligonucleotides indicated that the insert was in the correct orientation whereas PCR products obtained using the bottom strand

oligonucleotides indicated that the insert was in reverse orientation. Insert-containing clones were sequenced using the Fos primer to confirm the number of multimers, their orientations, and the fidelity of the BamHI and BglII restriction sites.

DNA Sequencing. Nucleotide sequences of CsCl-purified or of mini-preparations of plasmids were determined by the dideoxy chain termination method using Sequenase Version 2 kits (United States Biochemical Corp., Cleveland, OH).

Transfection. Transfection was performed by electroporation using Cell ZapII (Anderson Electronics, Brookline, MA). Briefly, RPMI 1640 was used to wash and resuspend an appropriate number of cells. 50×10^6 cells was the maximum transfected in 1 ml. In the experiments involving transfection of the 5′ deletion constructs and the linker-scanning mutations, the internal control plasmid pSV2CAT (35) was added to the resuspended cells, mixed well, and 0.9 ml of the cell mixture was pipetted into sterile cuvettes. CsCl-purified plasmid DNAs were added in a volume of 100 μl to the cuvettes. The cells were electroporated at 1250 μF/300 V, rested at room temperature for 10 min, and pipetted into complete medium at $\sim 0.25 \times 10^6$ cells/ml. After addition of inducers, cells were incubated for 8–18 h and then assayed for luciferase or chloramphenicol acetyl transferase (CAT) activity.

Luciferase Assays. Luciferase assays were performed as described (36). Cells were washed in PBS, transferred to Eppendorf tubes, and lysed in 200 μl Triton X-100 lysis buffer (1% Triton X-100, 25 mM glycylglycine, pH 7.8 [Sigma Chemical Co.]), 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM dithiothreitol [DTT]) at room temperature. The reaction mixture contained 100 μl cell lysate, 350 μl luciferase assay buffer (25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM KH_2PO_4, 2 mM ATP, and 1.27 mM DTT), and 100 μl of 1 mM luciferin (Analytical Luminescence Laboratory, San Diego, CA) dissolved in distilled H_2O. The level of luciferase activity was determined with a Monolight 2010 luminometer (Analytical Luminescence Laboratory) using plasmid pSV2Luc (34) as positive control. The values obtained from mock transfected or lysis buffer control were subtracted as background.

CAT Assays. The activity of cotransfected pSV2CAT was assayed to control for transfection efficiency by the diffusion-based assay (37). 50 μl of cell lysates was heated at 70°C for 10 min before mixing with 200 μl of 120 mM Tris, pH 7.8, 1 mM chloramphenicol (Sigma Chemical Co.), and 0.1 μCi [H^3]-acetyl CoA (200 mCi/mmol) (DuPont NEN Research Products, Wilmington, DE). The reaction mixture was added to a scintillation vial and 5 ml of Econofluor (DuPont NEN Research Products) was overlaid. The acetylated chloramphenicol was measured at the end of a 3-h incubation at 37°C.

Oligonucleotide Probes for Electrophoretic Mobility Shift Assay. Double-stranded oligonucleotides were generated by annealing a complementary pair of oligonucleotides. The reaction mixture contained 100 ng/μl of each oligonucleotide in 100 mM NaCl, 10 mM Tris-HCl, pH 8, and 1 mM EDTA. The DNA was incubated at 95°C for 10 min to disrupt secondary structures and incubated at 10°C below the melting temperature for 1 h and then slowly cooled to room temperature to anneal. The annealed oligonucleotides were ethanol precipitated and purified on a 12% polyacrylamide gel. Double-stranded oligonucleotides were 3′ endlabeled with Klenow enzyme and α[^{32}P]dCTP (3,000 Ci/mmol) to 5×10^8 cpm/μg, and gel purified as above.

Preparation of Nuclear Extracts. For small scale preparations, the method of Schreiber et al. (38) and for large scale preparations that of Dignam et al. (39), as modified by Boothby et al. (40) were used. Several protease inhibitors were added to all solutions just before use: 0.5 mM DTT, 0.5 mM PMSF, aprotinin (2 μg/ml), chymostatin (25 μg/ml), and leupeptin (10 μg/ml) (Boehringer Mannheim). Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Richmond, CA).

Electrophoretic Mobility Shift Assay. DNA binding reactions were performed in 20-μl reaction volumes containing 0.1 ng (30,000 cpm) labeled DNA probe, 2–5 μg nuclear extracts, 2–4 μg poly (dI-dC) (Pharmacia, Piscataway, NJ), 10% vol/vol glycerol, 50 mM KCl, 0.1 mM EDTA, 0.005% NP 40, 1 mM DTT, and 12.5 mM Hepes, pH 7.5. The amount of probe, poly (dI-dC), nuclear extract, and KCl concentration used was optimized for each fragment. The reaction mixture was incubated at room temperature for 30 min and then loaded onto a 4–6% native polyacrylamide gel. All gels were electrophoresed in recirculating 0.5× TBE buffer at 100 V for 2–3 h. Supershift experiments were performed by adding 1 μl of antiserum in a 20-μl binding reaction volume.

Results

Induction of Germline ε Transcripts in Two Mouse B Cell Lines. For these studies, we used two B lymphoma cell lines that inducibly express germline ε RNA. The I.29μ mouse B lymphoma expresses surface IgM and can be induced to undergo class switching to IgA by treatment with LPS. Switching can be increased by treatment with TGF-β, which increases transcription from the unrearranged Cα genes. I.29μ cells will switch to IgE expression if treated with IL-4 plus LPS, although switching to IgE is about 100 times less frequent than optimal switching to IgA. The maximal frequency of cells expressing IgE (both cytoplasmic and surface expression) at day 5 after induction is ~0.1%, but this level can increase by day 7 to ~0.5% (18, and data not shown). I.29μ cells synthesize germline ε transcripts constitutively, and this can be further increased 5–20-fold by treatment with IL-4 (Fig. 1 *B*, *left*) (2). Addition of LPS does not further increase the level of transcripts. The other B cell lymphoma used for these studies is M12.4.1, a class II^+, sIg-negative variant that was derived from the M12 B lymphoma (20). M12.4.1 cells do not express germline ε transcripts constitutively, but germline ε transcripts can be induced by treatment with IL-4 plus LPS (Fig. 1 *B*, *right*) (13). Unlike, I.29μ, IL-4 alone does not induce detectible germline ε transcripts in M12.4.1 cells.

To begin to characterize DNA sequences that regulate transcription of germline ε RNA, we verified by a RNase protection assay the initiation sites of transcription of germline ε RNA that were previously determined in 18-81A20 cells by Rothman et al. (28). RNA from IL-4–treated I.29μ cells was hybridized with a radioactive RNA probe transcribed from a 512-bp HincII/PstI ε genomic DNA fragment, digested with RNase, and the products were electrophoresed on a DNA sequencing gel. Three protected fragments of 118, 100, and 90 nucleotides were detected, which were not detected when the probe was hybridized with yeast RNA (Fig. 1 *C*). Assuming that the Iε splice donor defined previously by analysis of cloned germline ε cDNA from 18-81A20 cells is also used in I.29μ cells, we identified three transcription start sites very near the cluster of initiation sites previously identified (28). The first is located six nucleotides 5′ to the most 5′

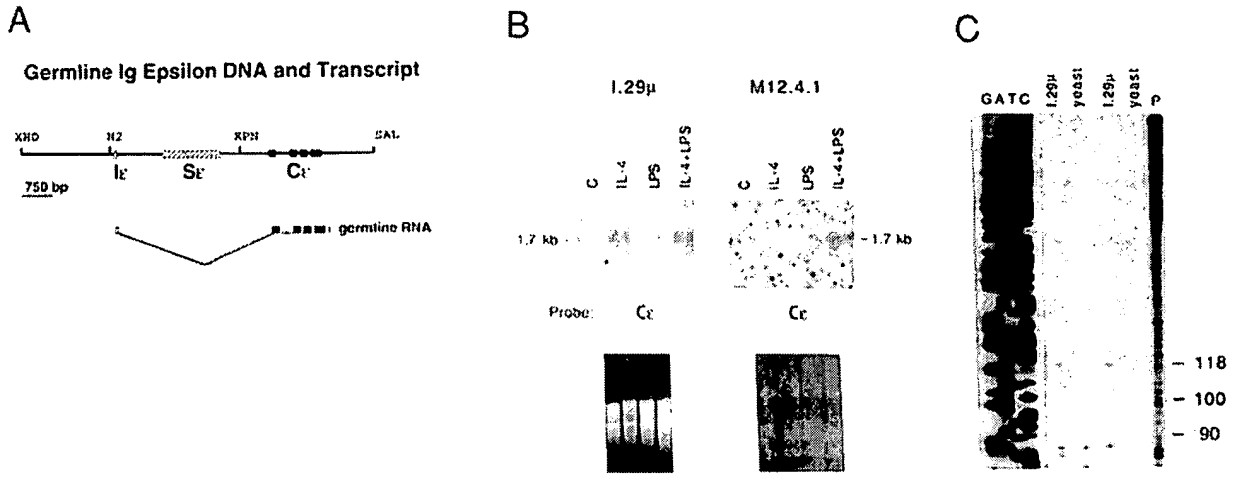

Figure 1. Structure of the mouse Ig heavy chain germline ε gene and ε transcript. (*A*) Restriction map of unrearranged Cε gene and germline ε transcript with location of exons and switch region (*Sε*) marked. (*B*) Blots containing total cell RNA from I.29μ (*left*) and M12.4.1 (*right*) cells that had been cultured for 2 d, as indicated, with medium alone, IL-4 (2,000 U/ml), LPS (25 μg/ml), or with both IL-4 and LPS, hybridized with Cε probe. (*C*) Products of an RNase protection experiment using a RNA probe transcribed from the 512-bp HincII/PstI genomic DNA fragment encompassing the Iε, hybridized with total cell RNA from I.29μ cells induced with X63-IL-4 supernatant at an optimal dose. Hybridization was at 42°C (*two left* lanes) or at 40°C (*two right* lanes). Three RNase resistant bands of the indicated lengths (determined by alignment with a DNA sequencing ladder) are detected on the DNA sequencing gel, and the positions of the corresponding RNA initiation sites are indicated on the sequence of the promoter-Iε-Luc construct in Fig. 2 *A*. We were unable to detect any band corresponding to a start site at −123, relative to our first start site, which was detected in I.29μ cells by primer extension experiments (53).

site identified in 18-81A20. The start sites are indicated by arrows on the sequence of the promoter in Fig. 2 *A*. The nucleotide positions referred to in this manuscript are all given relative to the first RNA initiation site in I.29μ cells.

Transient Transfection of Luciferase Reporter Plasmids Containing Various Lengths of 5′ Flanking Region from the Germline ε Segment. To identify DNA segments necessary for regulation of transcription of germline ε RNA, a series of luciferase reporter plasmids containing various lengths of the 5′ flank of the Iε exon were prepared, varying from having 1.8 kb to only 15 bp of 5′ flanking sequences. The ε promoter segment present in the plasmid named −162Luc is shown in Fig. 2 *A*. The plasmids were transiently transfected into I.29μ or M12.4.1 cells. Optimum luciferase activity was obtained when cells were harvested 12 h after transfection. An internal control CAT plasmid driven by a SV40 promoter/enhancer (pSV2CAT) was used to correct for transfection efficiency when comparing the basal activity of different plasmids. Maximal basal expression is not affected much by deletion of the 5′ flanking segment to −127 relative to the RNA initiation sites, but further deletion reduces basal expression in both I.29μ and M12.4.1 cell lines (Fig. 3, *A* and *B*: compare constructs *−162LUC*, *−127LUC*, *−115LUC*; and data not shown).

To test whether expression of these promoter-luciferase plasmids could be induced by IL-4, cells were divided after transfection into two more more aliquots and treated with recombinant mouse IL-4 for various times. Optimal responses were obtained with a dose of 240 ng/ml, which is equivalent to 2,500 U IL-4/ml, and we routinely used 1,000 U/ml. The IL-4 inducibility of plasmids with the longest 5′ flanks, having 1.8 and 1.2 kb of ε promoter segment, is two to four times less than plasmids having from 640 to 162 bp of 5′ flank, which are approximately equivalently inducible by IL-4: eightfold in I.29μ and threefold in M12.4.1 cells. Deletion past −162 reduces inducibility of the luciferase plasmids in both cell lines, and expression of a construct with 115 nucleotides of the ε promoter segment cannot be induced by IL-4 (Fig. 3, *C* and *D*; and data not shown). Therefore, the DNA segment residing between −162 and the start site of transcription has one or more DNA elements required for IL-4 inducibility of the ε promoter luciferase plasmids.

Effects of a Series of Linker-scanning Mutations on Expression of the Germline ε Promoter. Linker-scanning mutations were introduced into the −162/+53 promoter segment contained within the −162Luc reporter plasmid in order to identify DNA sequences required for IL-4 inducibility and for basal activity of the germline ε promoter. The nucleotides affected by the mutations, either because of substitution or deletion, are shown in Fig. 2 *B*, and their positions are indicated to the left of each sequence.

Plasmids bearing the linker-scanning mutations were transiently transfected into I.29μ and M12.4.1 cell in order to test for their ability to drive luciferase expression. Each construct was tested at least three times in both cell lines. Fig. 3 *A* shows that most of the linker-scanning mutations reduce basal level expression of the promoter relative to that obtained with the wild-type −162Luc plasmid when transfected into I.29μ cells, although for most mutations, the effect is not dramatic. The effects of the mutations differ in M12.4.1 cells, in that nearly all mutations located 3′ to −87 abolish basal expression although mutations located 5′ to −92 do

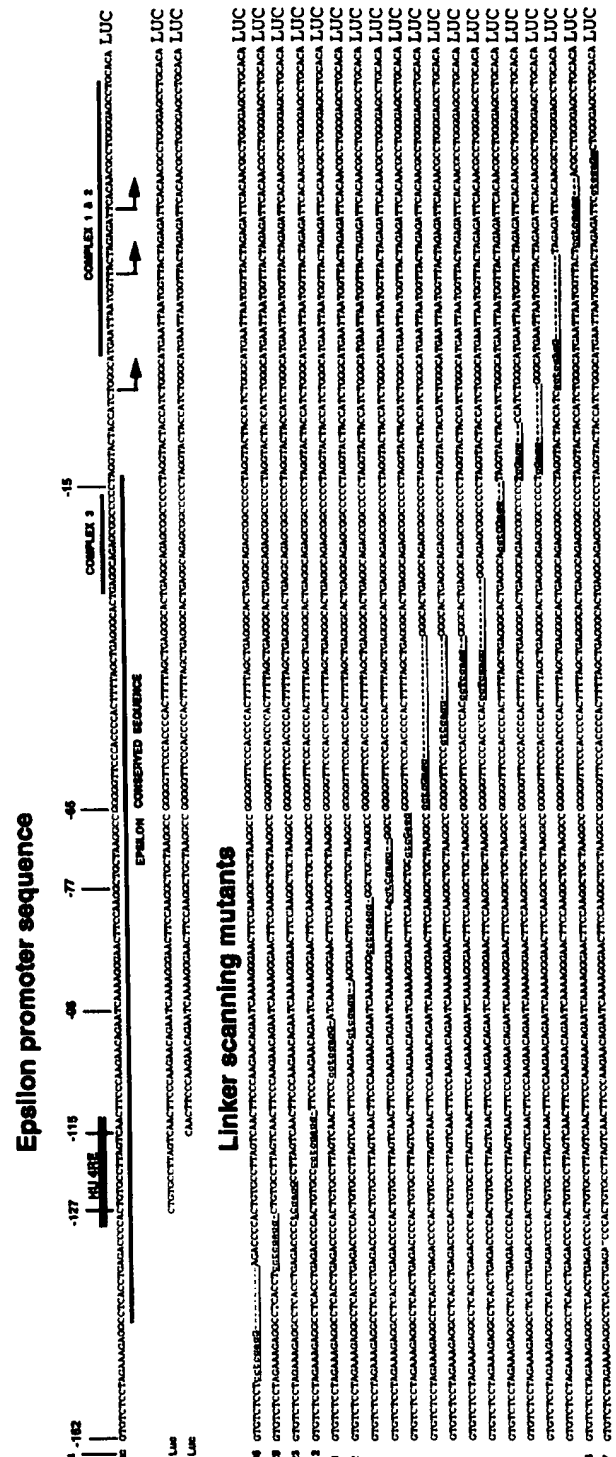

Figure 2. (*A*) Sequence of 5′ flank of germline ϵ RNA and the first 53 nucleotides of Iϵ exon present in the ϵ promoter −162Luc plasmid. This plasmid contains all three transcription start sites detected in I.29μ

not (Fig. 3 *B*). These results suggest there are several DNA elements residing between −136 and the start site of transcription that are necessary for normal levels of basal expression of the germline ϵ promoter in both cell lines, as assayed in the luciferase plasmid. Consistent with this is the fact that the nucleotide sequence between −144 and −14 is highly conserved, as it is almost identical to the sequence of the human germline ϵ promoter sequence (14, 41).

Localization of an IL-4RR with Linker-scanning Mutations. The sequences in the germline ϵ promoter which control induction by IL-4 appear to be much more localized than those required for basal expression, since only four of the linker-scanning mutations abolish or markedly reduce IL-4 inducibility in both I.29μ and M12.4.1 cells, mutant (mt) 13: at −120/−112, mt 20: at −106/−98, mt 27: at −87/−79, and mt 63: at −65/−39 (Fig. 3, *C* and *D*).

The sequence of the −126/−79 segment which contains three of the linker-scanning mutations that abolish or reduce IL-4 inducibility (mts: 13, 20, and 27) is shown in Fig. 4 *A*. Interestingly, these three mutations altered potential binding sites for three transcription factors. Mt 13 alters a consensus binding site for the C/EBP family of transcription factors (42–45). Mt 20 alters a consensus element for a newly described IL-4 inducible transcription factor, NF-IL4 or IL-4NAF (46, 47). Mt 27 alters a consensus binding site for NF-κB (48). This third mutation also abolishes constitutive expression. The fourth linker-scanning mutation mt 63 (−65/−38) reduces IL-4 inducibility in I.29μ cells by only 60% and basal expression by 90%, although it completely abolishes inducibility and basal expression in M12.4.1 cells. This mutation should abolish the binding site for the B cell–specific DNA binding protein BSAP, which binds to the germline ϵ promoter at −42/−15 and is required for IL-4 plus LPS inducibility of a CAT reporter gene driven by the mouse germline ϵ promoter in M12.4.1 cells (13, 15). Note, however, that four other mutations covering the region from −55 to −15 also mutated the BSAP binding site, but have little or no effect on induction by IL-4, although they all eliminate basal activity in M12.4.1 (but only slightly reduce it in I.29μ cells).

A DNA Segment Containing Binding Sites for C/EBP and NF-IL4 Is Sufficient to Confer IL-4 Inducibility. We wished to determine whether the DNA sequences shown by the linker-scanning mutations to be necessary for IL-4 induction

cells. The first 53-bp of the Iϵ exon does not have a translation initiation ATG codon. Regions indicated above the sequence are: (*Hu 4RE*) region necessary for induction of the human germline ϵ promoter by IL-4 (14); (*complex 1 & 2*) a region that binds nuclear factors in B cells/B cell lines stimulated by IL-4 (13): (*complex 3*) the binding site for BSAP (15, 54) and necessary for expression of the germline ϵ promoter-CAT constructs after LPS plus IL-4 induction (13, 15). (*EPSILON CONSERVED SEQUENCE*) The segment is highly homologous (>80%) between human and mouse sequences. This sequence differs at two sites from that previously reported (28): at +40/+43 we find GGGG instead of GGG and at −15/−19 CCCCC instead of CCCC. (*B*) Sequences of the −162Luc plasmids with linker scanning mutations. Names are given on the left, followed by nucleotides that are either substituted or deleted in the various mutants. These sequence data are available from EMBL/GenBank/DDBJ under accession number U17387.

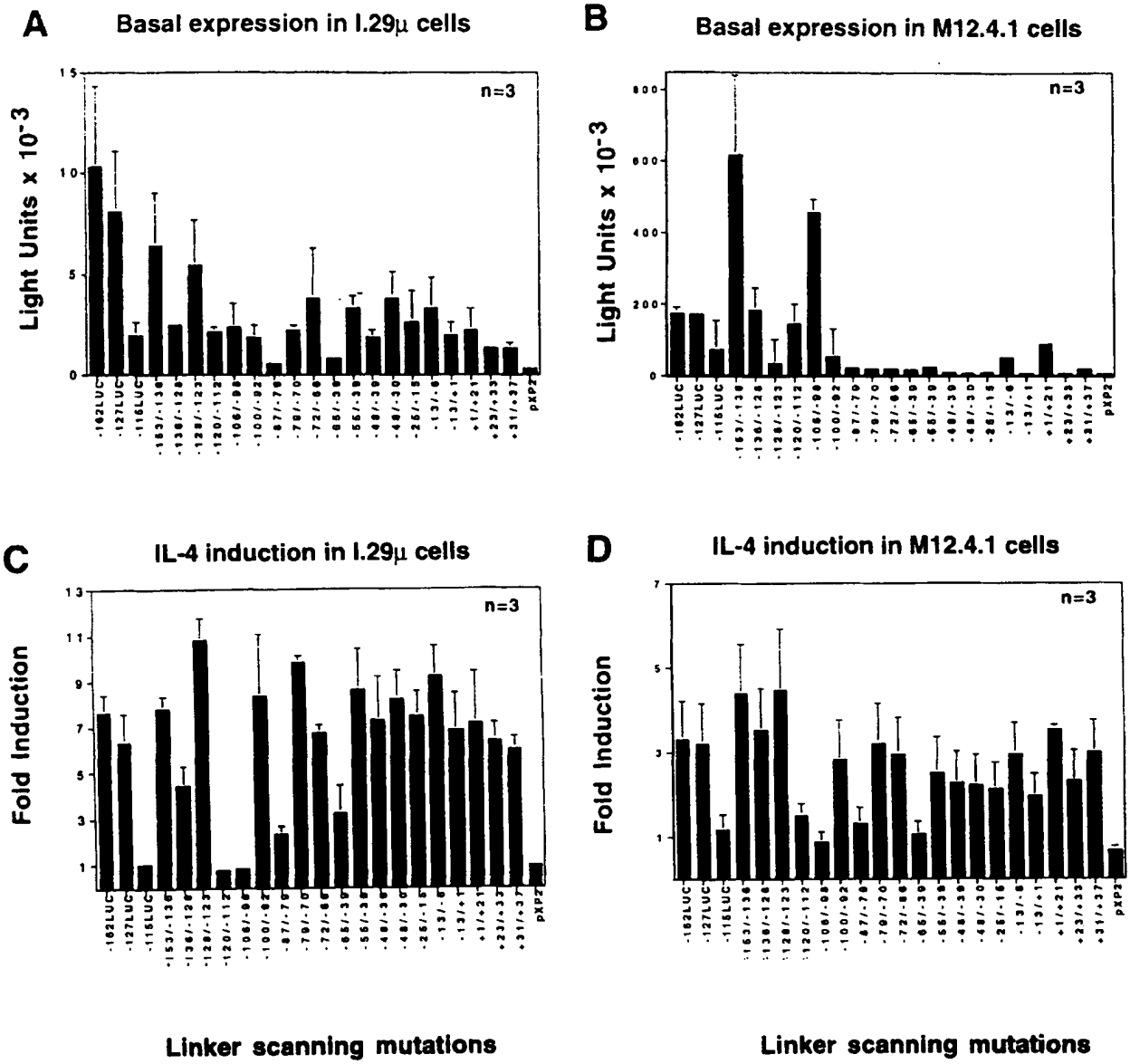

Figure 3. Results of transient transfection experiments of germline ϵ promoter-luciferase reporter plasmids having 5′ deletions: −162Luc, −127Luc, and −115Luc, or −162Luc with the indicated linker scanning mutations. (*A*) Basal expression in I.29μ. Luciferase activity is reported in light units after subtraction of background (no cell extract) of 250–350 light units. Results are normalized to the activity of pSV2CAT which was cotransfected along with the luciferase plasmids. Results from three experiments plus standard deviations of the means are plotted. (*B*) Basal expression in M12.4.1, as in A. (*C*) IL-4–inducible expression in I.29μ. Transfected cells from experiments shown in A were split into two aliquots, one of which was treated with IL-4 for 12 h. Fold induction indicates the luciferase activity in IL-4–treated cells relative to that in untreated cells. (*D*) IL-4–inducible expression in M12.4.1, as in C.

of the germline ϵ promoter are also sufficient to confer IL-4 inducibility upon a heterologous promoter. To test this, we inserted a series of double-stranded oligonucleotides containing wild-type or mutated sequences of the IL-4RR shown in Fig. 4 *A* into a luciferase reporter plasmid driven by a minimal c-fos promoter. Some of the plasmids have multiple copies of the oligonucleotides in sense and antisense orientations (indicated in Fig. 4 *B*). The plasmids were transiently transfected into I.29μ cells, and the cells were stimulated with IL-4. Only the wild-type oligonucleotide A, which contains both the consensus binding sites for C/EBP and NF-IL4, is able to confer IL-4 inducibility upon the c-fos promoter (Fig. 4 *B*). The activity of a plasmid with one copy of the A oligo is induced threefold by IL-4, and plasmids with two or three copies are induced about 20-fold. If either the C/EBP or the NF-IL4 site is mutated, the plasmid is not inducible by IL-4. Therefore, both sequence elements are required for IL-4 inducibility, consistent with the linker-scanning anal-

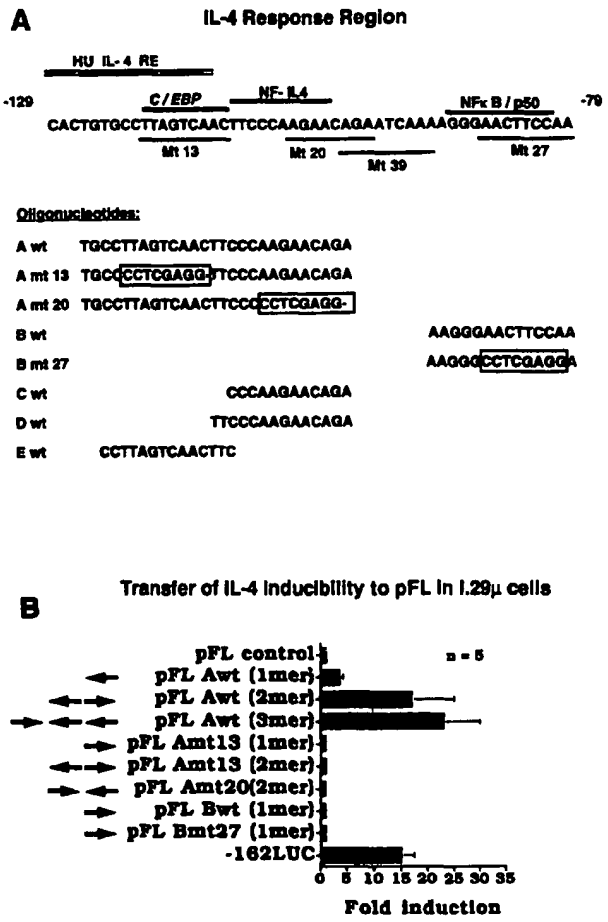

Figure 4. (*A*) Nucleotide sequence of the IL4RR in the mouse germline ϵ promoter. Three transcription factor binding sites are detected: a C/EBP consensus element (−120/−112), the sequence defined as an NF-IL4 site (−111/−102), and a NF-κB/p50 site (−90/−81). Indicated below the DNA sequence are the positions of the linker scanning mutations tested. (*Hu IL4 RE*) The segment which when mutated disrupts IL4 responsiveness of human germline ϵ promoter-CAT constructs (14). Beneath the sequence are the wild-type and mutated double stranded oligonucleotides that were tested for the ability to transfer IL-4 inducibility to a minimal c-fos promoter and/or to bind to nuclear proteins in an EMSA. The mutations of the oligos are the same as those present in the linker scanning mutations. (*B*) Results of transient transfection experiments in I.29μ cells, testing the IL-4 inducibility of plasmids in which the indicated oligonucleotides were inserted upstream of the minimal 71-bp c-fos promoter driving a luciferase gene. Transfection and induction conditions were as described above. (*pFL control*) The vector control, which is not inducible by IL-4. The average and standard deviations of the means of five experiments are plotted. The plasmids tested are indicated by, for example, pFL Awt (*1mer*) which contains one copy of the wild-type A oligonucleotide. (*Arrows, left*) The orientations of the oligonucleotide(s) in each plasmid. (−162LUC) The wild-type germline ϵ promoter luciferase reporter plasmid.

ysis, in which it was found that mutation of either of these sequence elements eliminates inducibility. The wild-type B oligo, which has the consensus κB site, does not transfer IL-4 inducibility to the c-fos promoter. Results are similar when the plasmids are transfected into M12.4.1 cells, although the induction by IL-4 is not as great as in I.29μ cells (data not shown).

Electrophoretic Mobility Shift Assays Demonstrate that the IL-4RR Does Indeed Contain Binding Sites for NF-IL4, Ig/EBP-1, and NF-κB/p50. To determine if these consensus binding sites bind the predicted proteins, a series of electrophoretic mobility shift assays (EMSAs) were performed using the double stranded oligonucleotides shown in Fig. 4 *A* as probes and/or as competitors. When wild-type oligonucleotide A is incubated with nuclear extracts from unstimulated or IL-4–treated I.29μ cells, a low mobility complex is induced by IL-4 treatment (Fig. 5 *A*). Competition experiments demonstrate that this complex binds to a site that matches the consensus sequence for a complex induced by IL-4 (NF-IL4/IL-4NAF) in human monocyte and B cell lines (46, 47). This complex is competed by wild-type oligo A, by A mt 13, and by oligo D, but not by mt 20, which has nucleotide substitutions in the putative NF-IL4 binding site, or by oligo E (Fig. 5 *A*). Oligo C does not compete (data not shown), apparently because it lacks the first T of the binding site, which has been shown to be important for binding of NF-IL4 (46). A kinetic experiment showed that the IL-4 inducible complex is detected after 30 min of IL-4 treatment, is maximally induced by 4 h, and is maintained for 24 h (data not shown). No later time points were examined. The kinetics of induction are slower and more sustained than that found for the binding activity in monocytes (47).

The IL-4 inducible complex is also detected in splenic B cells treated with IL-4 alone for 12 h and LPS has no additional effect (Fig. 5 *C*). Thus, although LPS is required for induction of germline ϵ transcripts in splenic B cells, NF-IL4 can be induced by IL-4 alone, indicating that induction of NF-IL4 is not sufficient to induce transcription of the endogenous germline ϵ RNA.

MT 13 disrupts a consensus sequence for the C/EBP family of transcription factors. This family of proteins possesses a basic region and leucine zipper, and all members bind the same DNA sequence, although not all cells have all family members. B cells express two members of the family: C/EBPγ (originally called Ig/EBP-1), which is most abundant in pre-B cells, and C/EBPβ (also called NF-IL6, LAP, AGP/EBP, CRP-2, or IL6DBP), which is expressed in mature B cell lines and in splenic B cells induced with LPS (45). To determine if C/EBP family members bind the consensus C/EBP site, we tested binding of recombinant mouse Ig/EBP-1 (49). Both wild-type oligonucleotide A and mt 20 bind Ig/EBP-1, whereas mt 13 does not (Fig. 5 *B*). Thus, oligonucleotides with a wild-type, but not a mutated, consensus site for C/EBP bind Ig/EBP. The arrow on the left side of Fig. 5 *A* indicates a complex that might correspond to a C/EBP family member present in I.29μ, since it is competed with both oligos A and mt20, but not with mt 13 or oligonucleotide D. Furthermore, this complex is competed by a multimerized C/EBP binding site from the Ig μ intron enhancer (data not shown) (49). However, it is not competed by oligo E, which includes only the C/EBP site, suggesting that binding may require additional nucleotides (Fig. 5 *A*). We demonstrated that the putative κB site binds a member of the NF-κB/rel family of transcription factors by performing EMSAs using oligonucleotide B as a probe with nuclear extracts from untreated

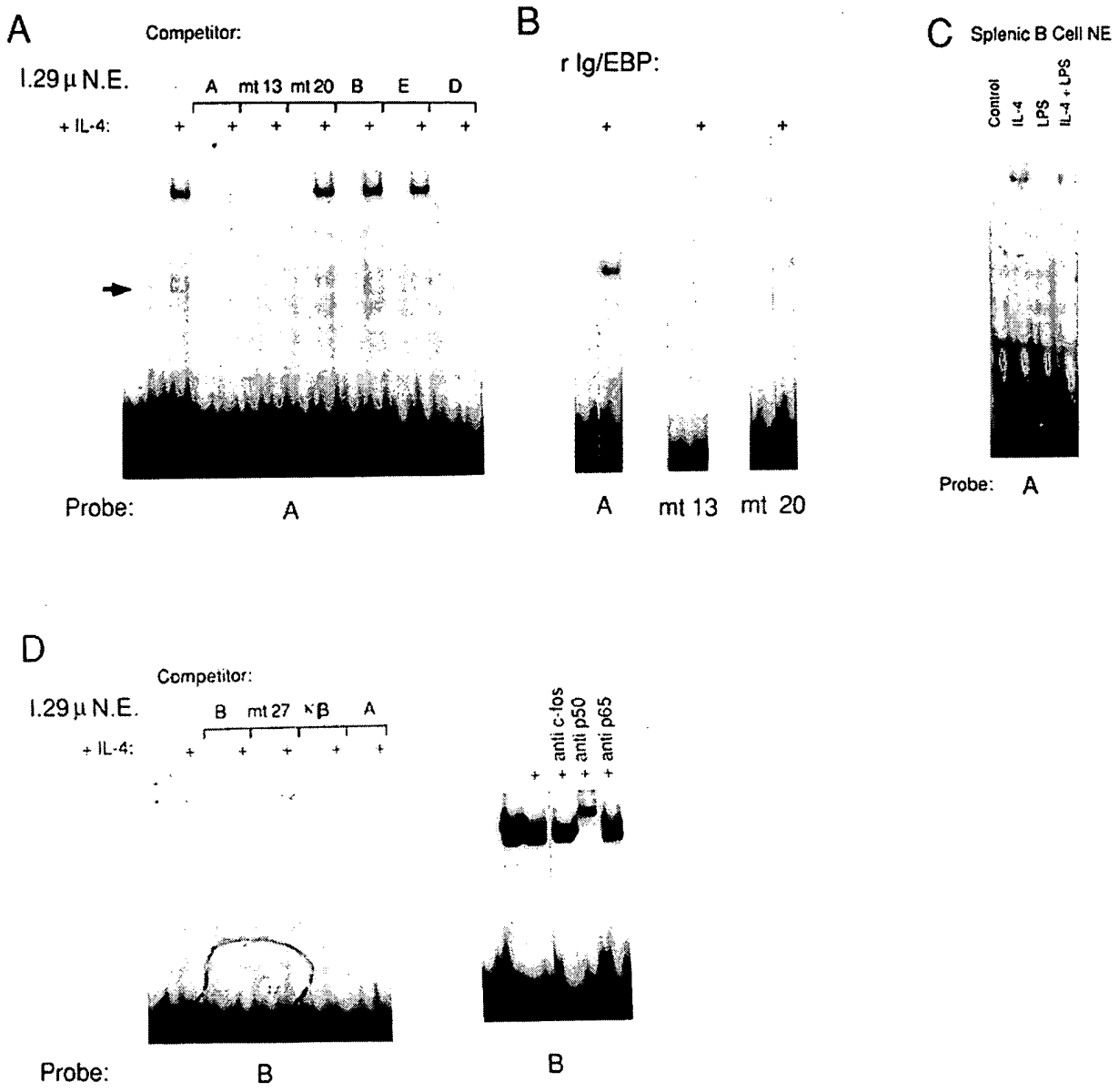

Figure 5. EMSAs of the IL-4RR within the germline ϵ promoter. Left-most lane in each panel or each set of lanes contains probe alone. (*A*) EMSA of oligonucleotide A (sequence given in Fig. 4 *A*) incubated with 5 μg nuclear extracts from I.29μ cells untreated or induced for 12 h with IL-4 (+). Competitor oligonucleotides were added at 100-fold molar excess. (*Arrow*) A complex that appears to be due to binding at the C/EBP site (see text). (*B*) Complexes formed after incubation of recombinant Ig/EBP-1(C/EBPγ) (+) with the wild-type A oligonucleotide, with mt 13 or with mt 20. (*C*) Oligonucleotide A incubated with nuclear extracts (2 μg) from splenic B cells (*Control*) or splenic B cells treated with IL-4, LPS, or IL-4 plus LPS for 12 h. (*D*) EMSAs with oligonucleotide B demonstrate that NF-κB/p50 binds to the wild-type B oligonucleotide. (*Left*) Nuclear extracts from untreated or IL-4–treated I.29μ cells (+) were incubated without or with the indicated oligonucleotides as competitors. κB is a 27-bp oligonucleotide containing the κ enhancer κB site. (*Right*) Supershift experiment using three antisera. Antiserum to NF-κB/p50 causes a supershift, but antisera for c-Fos and NF-κB/p65 do not (Santa Cruz Biotechnology, Santa Cruz, CA). Antisera specific for p50 and p65 from Dr. Nancy Rice (Frederick Cancer Center, Frederick, MD) gave identical results (data not shown).

or IL-4–treated I.29μ cells, competing with a DNA fragment containing the NF-κB site from the Ig κ enhancer (Fig. 5 *D*). Specificity of binding was also demonstrated by competition with wild-type B or with mt 27, which contains the mutated κB site. All three complexes formed with oligo B can be competed with the wild-type B oligo or with a κB binding site, but not with mt 27. In addition, when mt 27 is labeled and used as a probe it fails to bind any complex (data not shown). The binding is not induced by IL-4. This is consistent with the finding that oligo B does not transfer IL-4 inducibility to the c-fos promoter, although the κB site contributes to the IL-4 response, since the linker-scanning

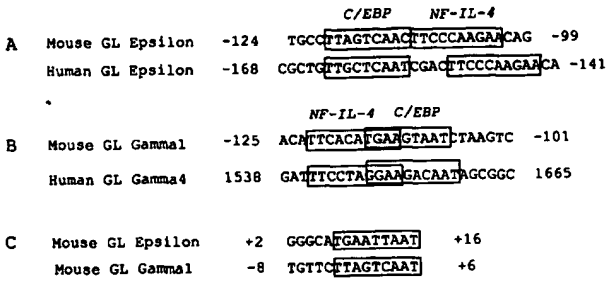

Figure 6. Comparison of DNA sequences 5' to the I exons of IL-4 responsive mouse and human germline C_γ genes. (*A*) Alignment of the mouse and human germline ϵ promoters to show homology between the C/EBP and NF-IL4 sites. (*B*) Alignment of NF-IL4 and C/EBP sites in the mouse germline $\gamma 1$ promoter with sequences upstream of the human $\gamma 4$ switch region. The C/EBP site in the $\gamma 1$ promoter has been demonstrated to bind C/EBPγ and C/EBPβ in nuclear extracts (17). The positions of the nucleotides from the $\gamma 4$ gene are according to the published sequence (41). (*C*) Alignment of the C/EBP consensus sequences located at the start sites of transcription of the mouse germline ϵ and $\gamma 1$ RNAs. The $\gamma 1$ sequence has been shown to bind C/EBPγ in nuclear extracts (17).

mutation at this site reduces the response to IL-4 by threefold in both I.29μ and M12.4.1 cells. Different members of the NF-κB/rel family differ somewhat in their DNA sequence requirements for binding (48). To determine which member of this family binds to the putative binding site at $-90/-81$, we tested a series of NF-κB antisera for their effect in EMSAs using oligo B as the probe. An antiserum to the p50 homodimer supershifts the complex, whereas antisera to p65 or p50B do not (Fig. 5 *D*, *right*, and data not shown). As a further control, we show that anti c-Fos antibody does not supershift the complexes. Note that linker-scanning mutation 39, which mutates $-100/-91$, has no effect on expression of the promoter in these assays, suggesting that there is no additional transcription factor binding site between the sites for NF-IL4 and for NF-κB/p50.

Discussion

Results Reported Here Extend Previous Studies of the Regulation of the Ig Germline ϵ Promoter in Mouse and Human. The regulation of the mouse germline ϵ promoter by LPS plus IL-4 has been previously analyzed using a CAT reporter plasmid transiently transfected into M12.4.1 cells (13). Rothman et al. (13) found that a 5' deletion that deleted the C/EBP site did not reduce inducibility of their reporter gene, whereas we find that mutation of the C/EBP site abolishes induction by IL-4 when used in the absence of LPS. Furthermore, they found that the BSAP binding site is essential for LPS plus IL-4 inducibility in M12.4.1 cells (15), whereas we find that four out of five mutations that affect the BSAP binding site in the promoter do not reduce IL-4 inducibility in either M12.4.1 or I.29μ cells. Thus, it is possible that induction by IL-4 alone may utilize different transcription factor binding sites than the combination of IL-4 plus LPS. Our findings agree with preliminary results on the IL-4RR of the promoter for the human germline ϵ transcripts (14).

Characterization of an IL-4RR in the Germline ϵ Promoter. We have identified three transcription factors that appear to regulate basal and IL-4 induction of transcription of the mouse germline ϵ promoter: one or more members of the C/EBP family of transcription factors, the p50 subunit of NF-κB, and a newly described IL-4–inducible transcription factor termed NF-IL4 or IL-4 NAF (46, 47). We demonstrate that mutation of any of the sequence elements that bind these factors, within the context of the germline ϵ promoter, abolishes or reduces induction by IL-4. A fragment containing binding sites for C/EBP and NF-IL4 is sufficient to transfer IL-4 inducibility to a minimal c-fos promoter, but a fragment containing only the C/EBP consensus element or only the NF-IL4 element is not sufficient. The binding sites for these three factors and their positions relative to each other are conserved within the human germline ϵ promoter. Fig. 6 *A* shows an alignment of the C/EBP and NF-IL4 consensus elements in the mouse and human germline ϵ promoters. Furthermore, substitutions in the C/EBP consensus element of the human germline ϵ promoter eliminate IL-4 responsiveness (14). Effects of mutations in the NF-IL4 or NF-κB consensus elements of the human promoter have not been tested.

Consensus binding sites for the IL-4–inducible complex NF-IL4/IL-4NAF have been identified in various IL-4–inducible promoters (46, 47). Although Kohler and Rieber (46) demonstrated that mutation of the binding site reduced the IL-4 inducibility of a CD23 promoter transfected into a human B cell line, no evidence has been presented to indicate that the binding site for this factor by itself can transfer IL-4 responsiveness to another promoter.

Interestingly, binding sites for C/EBP and NF-IL4 are also found in the promoter for mouse germline $\gamma 1$ RNA at about the same distance from the first RNA initiation site ($-122/-108$) as in the ϵ promoter. The segment containing these binding sites is necessary for induction of the promoter-luciferase reporter plasmids by phorbol ester and IL-4 (16). Furthermore, both C/EBPβ and C/EBPγ have been shown to bind the C/EBP site in the germline $\gamma 1$ promoter (17). Unlike the ϵ promoter, the germline $\gamma 1$ promoter is not induced by IL-4 alone, although IL-4 synergizes with phorbol ester to activate this promoter. Fig. 6 *B* shows that in the $\gamma 1$ promoter the C/EBP and NF-IL4 elements overlap and have a different position relative to each other than in the ϵ promoter. Switching to IgG4 in humans is inducible by IL-4, but the germline $\gamma 4$ promoter and transcription start site have not been defined. These same two consensus elements are present within an evolutionarily conserved region 5' to the S$\gamma 4$ tandem repeats, which has been postulated to encode the I$\gamma 4$ exon and its promoter (41) (Fig. 6 *B*). Thus, it appears possible that the proteins that bind the C/EBP and NF-IL4 sites interact and function together to effect IL-4 inducibility in all four of these promoters. Although we have been unable to detect a complex of these factors in the EMSAs with oligo A, results suggestive of such an interaction are shown in Fig. 5. In Fig. 5 *B* it appears that the mt 20 A oligo binds Ig/EBP-1 less well than the wild-type A oligo, although the nucleotides mutated in this oligonucleotide do

not overlap the C/EBP consensus site. This suggests that Ig/EBP may also bind the NF-IL4 site. This is consistent with the finding in Fig. 5 *A* that oligo E, which contains only the C/EBP site, does not compete with the putative C/EBP complex formed with oligo A.

An additional C/EBP consensus element is present at the start site of transcription in both the mouse ϵ and $\gamma 1$ germline promoters (17) (see Fig. 6 *C*). Lundgren et al. (17) have shown that C/EBP proteins bind at this second C/EBP site in the $\gamma 1$ promoter. The second C/EBP site in the ϵ promoter is located where an IL-4–inducible complex, termed STF-IL4, has been shown to bind by footprinting and by EMSA competition analyses (13, 50). Unlike the C/EBP sites located upstream in each of these promoters, no obvious consensus binding sites for NF-IL4, as previously defined (46, 47), are nearby. This is consistent with our finding that linker-scanning mutations in this region do not reduce IL-4 inducibility, although they do reduce or abolish basal activity in I.29μ or M12.4.1 cells, respectively.

C/EBPβ/NF-IL6 has been demonstrated to interact (via its leucine zipper) with RelA (NF-κB/p65) and to synergistically activate transcription when binding the IL-8 promoter. By contrast, NF-κB/p50, when bound to the same κB site, had little or no ability to synergize with C/EBPβ (51, 52). We have been unable to detect binding of p65 to the κB site in the ϵ promoter, although binding of p50 is readily detected. An attractive hypothesis is that induction of NF-IL-4 by IL-4 aids the interaction between C/EBPβ and p50, thus creating an effective transcriptional activator involving C/EBPβ, NF-κB/p50, and NF-IL4. Our data suggest that p50 may interact with a C/EBP protein and/or with NF-IL4, since we have shown that mutation of the κB/p50 site reduces IL-4 responsiveness by threefold, although fragment B, which contains the κB site but not the C/EBP or NF-IL4 sites, is not sufficient to transfer IL-4 inducibility to a minimal c-fos promoter.

In conclusion, we have defined and characterized an IL-4RR in the promoter for germline ϵ RNA which is necessary for induction of the promoter by IL-4 when assayed in a luciferase reporter plasmid. This IL-4RR is sufficient to transfer IL-4 inducibility to another promoter as long as binding sites for NF-IL4 and C/EBP transcription factors are both intact. Binding sites for these same two transcription factors are also closely spaced in promoters of other germline transcripts inducible by IL-4. Future studies will be directed towards determining if these factors form a complex that effects IL-4 induction.

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Note added in proof: Since the submission of our manuscript an additional report on the IL-4 response region of the human germline ϵ promoter which is mostly consistent with our results has been published: Albrecht, B., S. Peiritsch, and M. Woisetschlager. 1994. A bifunctional control element in the human IgE germline promoter involved in repression and IL-4 activation. *Int. Immunol.* 6:1143–1151.

In addition, the protein IL-4 Stat, the cDNA for which has recently been cloned, appears to correspond to the complex we refer to as NF-IL4 in our manuscript. The reference is Hou, J., U. Schindler, W. J. Henzel, T. C. Ho, M. Brasseur, and S. L. McKnight. 1994. An interleukin-4–induced transcription factor: IL-4 Stat. *Science (Wash. DC).* 265:1701–1706.

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APPENDIX B

The human IgE germline promoter is regulated by interleukin-4, interleukin-13, interferon-α and interferon-γ via an interferon-γ-activated site and its flanking regions

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Class switching to IgE is preceded by the appearance of ε germline transcripts, which are induced by Interleukin-4 (IL-4) and by IL-13. A 51-bp fragment of the human ε germline promoter conferred in reporter gene assays with the erythroleukemic cell line TF-1 upregulation of transcription by IL-4 or IL-13, and repression by interferon-α (IFN-α) and IFN-γ. A central IFN-γ activated sequence within the 51-bp fragment was sufficient for transcriptional regulation by the cytokines in the absence of its normal flanking regions. In contrast, deletion of either upstream or downstream sequences abolished repression by IFN-α or INF-γ, but not upregulation by IL-4 or IL-13. IL-4 stimulated reporter gene transcription required more than ten times higher concentrations than cell proliferation or tyrosine phosphorylation of the IL-4 receptor.

Keywords: interleukin-4; interleukin-13; interferon, IgE antibody; class switching.

Activated B-cells proliferate and develop into plasma cells, which produce antigen-specific antibodies. A decisive step in this differentiation process is antibody class switching, which results in the sequential expression of antibodies with the same antigen-specificity, but different F_c parts [1]. Class switching to particular antibody types is preceded by the production of RNA transcripts which are initiated from a promoter upstream of the actual switch site. These transcripts contain a unique start exon, the I exon, which is spliced to the C_H coding exons. They are called germline transcripts, or sterile transcripts, because they are apparently never translated, due to the presence of stop codons in all reading frames [1]. The production of germline transcripts is regulated by two signals, one delivered by CD40 upon binding to its ligand, and the other provided by cytokines, which regulate the specificity of switching. A well documented example is interleukin-4 (IL-4), which induces germline transcripts starting from several initiation sites downstream of the Cε promoter [1–3]. Actual switching occurs usually by looping out the intervening DNA sequences [4]. Germline transcripts are thought to contribute to this process by regulating the accessibility of the switch regions [1].

Induction of IgE synthesis is strictly dependent on stimulation by either IL-4, or the related cytokine IL-13 [2, 5, 6]. Antibodies of the IgE subclass are produced in allergic diseases like hay fever, asthma or anaphylactic shock, and during infections with helminthic macroparasites. IgE molecules bind to high-affinity receptors on the surface of mast-cells and basophils. Antigen binding to these mast cell-associated antibodies triggers the release of allergic mediators like histamine or prostaglandins. Therapeutic modulation of IgE production may therefore be beneficial in allergic diseases and parasitic infections.

A number of control elements have been described in the Cε promoter from human and mouse [7–15]. Here we focus on a stretch of 51 bp with three adjacent sequences known or suspected to be involved in regulation by cytokines. We have studied these elements separately or combined in the context of a heterologous promoter.

The IL-4-responsive region of the Cε promoter was investigated by Ichiki et al. in a human B-cell line [9]. The region between −157 and −96 from the most upstream transcription-initiation site of IgE germline transcription [14] (i.e. −215 to −154 from the start site used as reference by Ichiki et al. [9]) was necessary for IL-4-induced transcription. Mutation analysis located the essential region to 12-bp between −105 and −94, and an oligonucleotide representing this region competed for IL-4-induced protein binding with a larger promoter fragment. The 12-bp sequence was designated IL-4RE, for IL-4-responsive element [9]. There is some similarity to the Y box from the promoter of MHC (major histocompatibility complex) class II genes [16] and to the CCAAT/enhancer-binding protein (C/EBP) consensus sequence [9]. It was shown by competition experiments in electrophoretic mobility-shift assays that one or more members of the C/EBP family bind to a sequence within this IL-4RE in mouse B cells [14, 15].

Just downstream of the IL-4RE is a region which fits the consensus for interferon-γ(IFN-γ)-activated sequences, or GAS sites. These elements were first described for IFN-γ, but many other cytokines also signal through GAS [17, 18]. Cytokines induce activation of Stat proteins (signal transducers and activators of transcription), which form complexes and migrate to the

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Abbreviations. BSAP, B cell specific activator protein; CAT, chloramphenicol acteyl-transferase; [D124]IL-4, mutant of IL-4 with Tyr124 replaced by Asp. [D121, D124]IL-4, double mutant of interleukin-4 with Arg121 and Tyr124 both replaced by Asp; GAS, IFN-γ-activated sequence; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IL-4RE, IL-4 responsive element; ISRE, interferon-stimulated response element; MHC, major histocompatibility complex; Stat, Signal transducer and activator of transcription; TK, Herpes Simplex virus thymidine kinase. C/EBP, CCAAT/enhancer-binding protein; NF-κB, nuclear factor κB.

nucleus, were they bind either GAS sites, or in combination with nuclear factors, to ISRE (interferon-stimulated responsive elements) [17]. IL-4 induces binding of a protein complex to the IgE germline promoter [10, 11, 15]. The binding complex contains Stat6, an IL-4-responsive and IL-13-responsive Stat protein [19]. A sequence between −97 and −82, which contains the GAS site, was necessary for IL-4-stimulated transcription in a Burkitt lymphoma line, and for suppression of transcription in the absence of IL-4 [13]. A single mutation within this element prevented both IL-4-dependent upregulation of transcription and IL-4-independent repression of transcription. Protein binding to the distal IL-4RE was constitutive and not IL-4-dependent in this system [13].

Downstream of the GAS site, between −77 and −62 is a sequence with similarity to ISRE sites [3]. It is suggestive that the promoters of the γ3 and γ4 germline loci in human, and of the ε, α and γ2b loci in mouse also contain potential ISRE sites [3]. The potential ISRE in the IgE germline promoter is highly conserved between human and mouse. Surprisingly, a factor identified to bind within this region is the p50 subunit of nuclear factor κB(NF-κB), rather than a protein associated with Stat signaling pathways [15].

Other IL-4RE have been identified in different locations of the mouse promoter [7, 8, 13, 20]. It is not clear whether these sites are involved in IL-4-induced transcription in human cells. We have studied various combinations of the distal IL-4RE, the GAS site and the potential ISRE in a heterologous reporter gene system. The results show that the central GAS confers regulation by IL-4, IL-13, IFN-α and IFN-γ, which is modulated and enhanced by the presence of flanking sequences.

Antagonistic variants of IL-4 have been created, which fail to signal because they cannot induce receptor dimerization [21–23]. We have tested whether the variants [D124]IL-4 and [D121, D124]IL-4, where one or two amino acids from the IL-4 signaling site have been replaced by Asp, could induce transcription in reporter gene assays.

MATERIALS AND METHODS

Cell culture, cytokines and antibodies. Cells were cultured in RPMI 1640 with 7% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2. For the erythroleukemic cell line TF-1, 33 ng/ml GM-CSF (granulocyte-macrophage colony stimulating factor) was added, while the monocyte-like histiocytic lymphoma U937 was grown without additions. Peripheral blood lymphocytes were obtained from lymphocyte concentrates of healthy donors by Ficoll centrifugation as described [24]. Cells (10^6/ml) were stimulated with 9 μg/ml phytohemagglutinin (Wellcome Diagnostics, Dartford, UK) for four days.

Proliferation was measured by [^{3}H]thymidine incorporation [24]. EC^{50} values for proliferation and phosphorylation were determined from at least three independent experiments, using the computer program GraFit (Erithacus Software).

Human IL-4, IL-4 variants, and GM-CSF were expressed in *Escherichia coli* and purified as described [25, 26]. Human IL-13 was a gift from Dr N. Vita (Sanofi Recherche, Labége, France); human IFN-α was from Biomol and human IFN-γ was a gift from Dr B. Otto (Fraunhofer Institute for Toxicology, Hannover, Germany).

The anti-(IL-4 receptor) mAb X14/38 was a gift of Drs P. Reusch and W. Sebald, Würzburg. The horseradish peroxidase-coupled recombinant anti-phosphotyrosine antibody RC20 (Affinity) was used for detection on western blots.

Northern blot analysis. Total RNA was isolated by the guanidinium thiocyanate/phenol/chloroform method [27]. The RNA was electrophoresed on a 1.2% formaldehyde gel with 15 μg total RNA/lane. After separation the RNA was transferred to a positively charged nylon membrane. Following fixation under calibrated ultraviolet irradiation, the membranes were hybridized with digoxygenin-labeled probes prepared using a DNA labeling kit (Boehringer). The blot was detected using alkaline phosphatase-labeled anti-digoxygenin antibody and a chemiluminescent substrate according to the manufacturers instructions (Boehringer, Mannheim). The application of equal RNA amounts was controlled by ethidium bromide staining.

Immunoprecipitation and western blotting. Cells were stimulated for 10 min with cytokines at 37°C and 5% CO_2, pelleted and suspended in ice-cold lysis buffer [28]. After spin-over rotation (30 min) the cell lysates were centrifuged at 10000×*g* for 10 min. Cleared supernatants were incubated for 2 h at 4°C with the anti-(IL-4 receptor) mAb X14/38, and immune complexes were collected on protein G-Sepharose for 1 h at 4°C. Precipitates were washed twice with lysis buffer, twice with salt buffer (0.5 M LiCl, 100 mM Tris/HCl. pH 8.0) and boiled for 2 min in SDS sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS, 26 mM dithiothreitol, 10% glycerol). The samples were subjected to SDS/PAGE on 7.5% gels followed by western Blot analysis with RC20 (0.1 mg/ml) using an enhanced chemiluminescence detection system (Amersham).

Reporter gene constructs. Promoter/enhancer constructs (E1–E8) containing DNA sequences from the Cε promoter were obtained by cloning chemically synthesized *SphI*/*Xba*I fragments between *SphI*/*Xba*I sites of the vector pBLCAT5 [29]. The following chemically synthesized oligonucleotides corresponding to regions upstream of the germline exon (Iε) of the Cε transcript were used for plasmid constructs E1 – E8. Nucleotides of endonuclease recognition sites and spacer nucleotides between the triplicate boxes of E1 and E4 are written in lower case:

E1, (−111 to −94)$_3$,
cCCGCTGTTGCTCAATCGAcCCGCTGTTGCTCAATCGAcCCGCTGTTGCTCAATCGACt
gtacgGGCGACAACGAGTTAGCTgGGCGACAACGAGTTAGCTgGGCGACAACGAGTTAGCTGagatc

E2, (−111 to −62),
cCCGCTGTTGCTCAATCGACTTCCCAAGAACAGAGAGAAAAGGGAACTTCCt
gtacgGGCGACAACGAGTTAGCTGAAGGGTTCTTGTCTCTCTTTTCCCTTGAAGGagatc

E3, (−111 to −77),
cCCGCTGTTGCTCAATCGACTTCCCAAGAACAGAt
gtacgGGCGACAACGAGTTAGCTGAAGGGTTCTTGTCTagatc

E4, (−98 to −80)$_3$,
cATCGACTTCCCAAGAACAGaATCGACTTCCCAAGAACAGaATCGACTTCCCAAGAACAGt
gtacgTAGCTGAAGGGTTCTTGTCtTAGCTGAAGGGTTCTTGTCtTAGCTGAAGGGTTCTTGTCagatc

E5, (−111 to −93),
cCCCGCTGTTGCTCAATCGACt
gtacgGGGCGACAACGAGTTAGCTGagatc

E6, (−98 to −80),
cATCGACTTCCCAAGAACAGt
gtacgTAGCTGAAGGGTTCTTGTCagatc

E7, (−98 to −63), cATCGACTTCCCAAGAACAGAGAGAAAAGGGAACTTCt
gtacgTAGCTGAAGGGTTCTTGTCTCTCTTTTCCCTTGAAGagatc

E8, (−77 to −63), cAGAAAAGGGAACTTCt
gtacgTCTTTTCCCTTGAAGagatc

Plasmids were analysed by digestion with restriction endonucleases and DNA sequencing of both strands. The constructs for transfection into TF-1 cells were purified by cesium chloride density-gradient centrifugation.

Transient transfection, stimulation and chloramphenicol actyltransperase (CAT) assays. Before transfection, TF-1 cells were cultured in fresh medium without GM-CSF for 24 h. For electroporation 7×10^6 cells in 350 µl complete medium (supplemented with 5% fetal calf serum) were mixed with 30 µg supercoiled plasmid DNA (in 50 µl 10 mM Tris, 1 mM EDTA, pH 7.4). The transfection was performed by a single pulse (250 V, 1200 MF, 34−39 ms) from a Eurogentec Easyject™- pulser. Subsequently, the cells were incubated in six-well tissue culture plates in medium containing GM-CSF (33 ng/ml) with or without various other cytokines (10 ng/ml) for 48 h. The cell extracts were prepared by a freeze/thaw procedure. Protein was determined according to Bradford [30] and CAT activity was assayed with the CAT ELISA kit from Boehringer.

TK (Herpes Simplex virus thymidine kinase) minimal promoter and the reporter gene CAT. Known elements and protein-binding sites within this IL-4-responsive region are summarized in Fig. 1. Various constructs were transfected into TF-1 cells, which express both IL-4Rα and γc (Fig. 2). These cells respond to IL-4 and IL-13 with short-term proliferation and transient tyrosine phosphorylation of IL-4Rα and other proteins [28, 31].

The TK minimal promoter stimulated a very modest expression of the reporter gene, which was enhanced by elements from the Cε promoter (Fig. 3). The complete 51-bp IL-4-responsive region induced nearly tenfold higher transcription compared to the TK promoter alone (construct E2). Addition of IL-4 increased CAT expression further by a factor of 2.5. All plasmids containing the GAS element in various combinations showed increased transcription and were responsive to IL-4 (Fig. 3). While the complete 51-bp fragment (E2) stimulated highest overall trancription, a construct with three copies of the GAS element (E4) showed the highest inducibility by IL-4, which upregulated transcription by a factor of four (Fig. 3).

RESULTS

To investigate the IL-4-responsive region of the human Cε promoter, we have coupled a synthetic 51-bp fragment to the

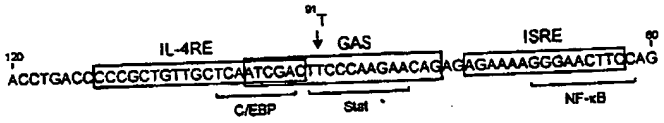

Fig. 1. IL-4-responsive region of the human IgE germline promoter, located between −120 and −60 upstream of the most upstream transcriptional-initiation site [14]. Three motifs of interest as well as binding sites for the transcription factors C/EBP, Stat6 and NF-κB/p50 are indicated. An exchange of residue −91 abolishes IL-4 responsiveness, as shown by Albrecht et al. [14]. References are provided in the text.

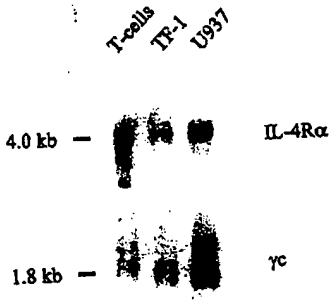

Fig. 2. IL-4Rα and γc expression in TF-1 cells as determined by northern blotting. Phytohemagglutinin stimulated T-cells and the monocytic cell line U937 are shown as controls.

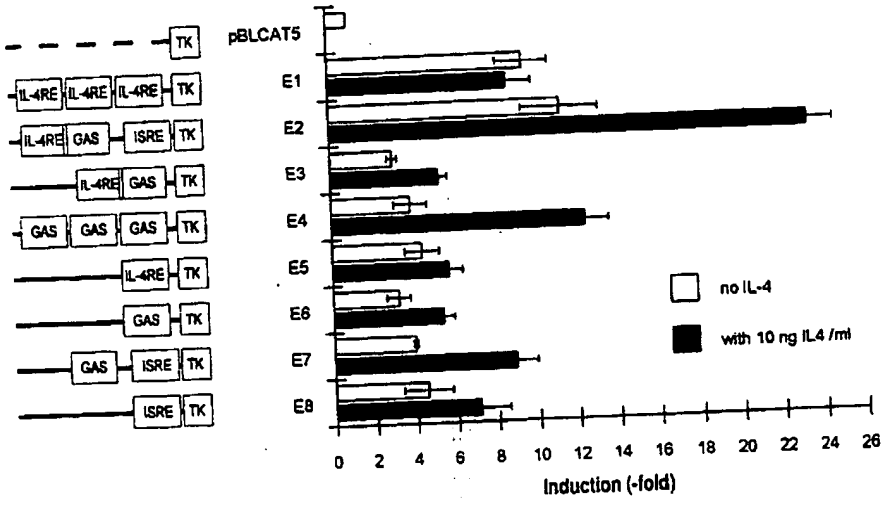

Fig. 3. Promoter–enhancer construct activities. A schematic map of promoter−enhancer constructs (E1−E8) coupled to the TK minimal promoter is shown on the left. Columns to the right represent relative CAT amounts synthesized during transient transfection experiments with TF-1 cells. Values shown are x-fold induction compared with the activity of the enhancerless TK vektor pBLCAT5 (= 1). cytokine. The data shown are averages of 3−6 independent experiments. Error bars indicate a 95% confidence interval ($\pm 2\sigma\sqrt{n}$).

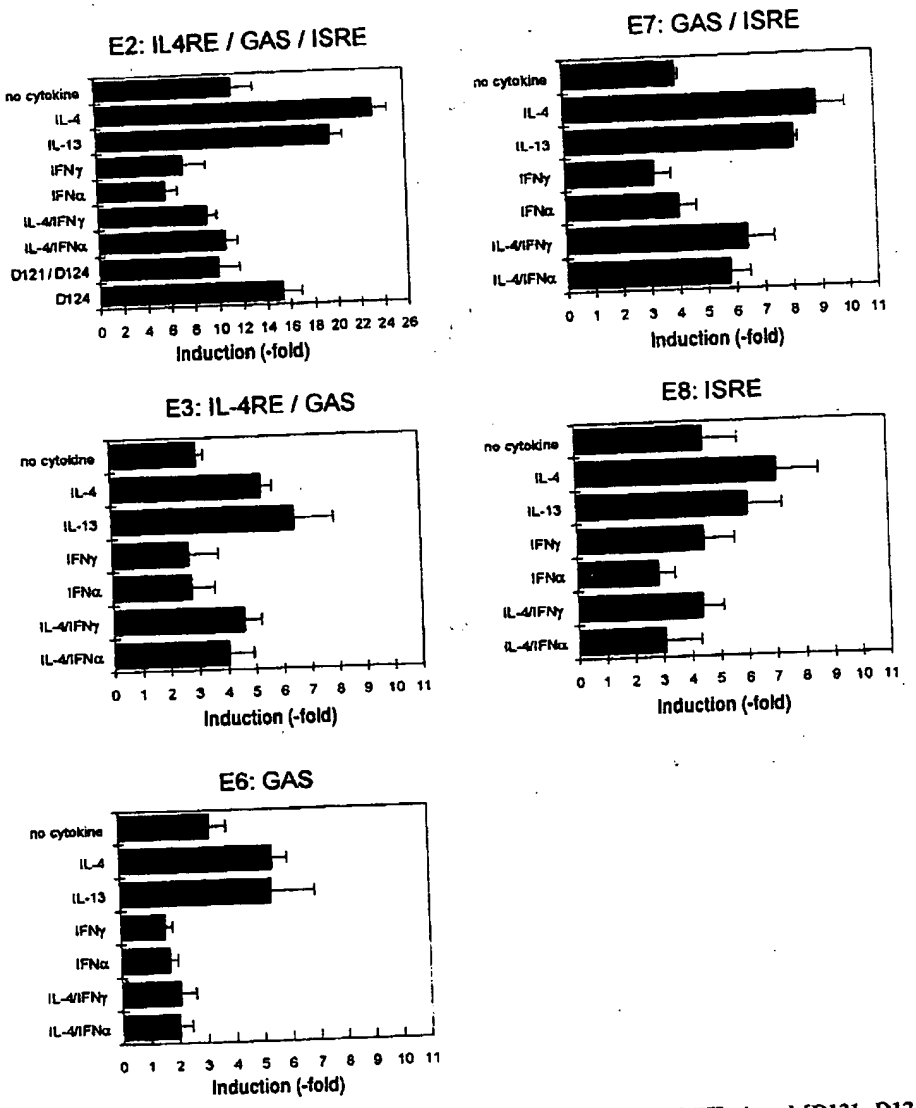

Fig. 4. Transcriptional activity of IL-4, IL-13, IFN-α, IFN-γ and the IL-4 antagonists [D124]IL-4 and [D121, D124]IL-4 for reporter constructs indicated, relative to the activity of unstimulated cells transfected with the enhancerless pBLCAT5 (= 1). The concentration was 10 ng/ml for each cytokine. The data shown are averages of 4–6 independent experiments. Error bars indicate a 95% confidence interval ($\pm 2\sigma\sqrt{n}$).

IL-4 induced little or no upregulation for the isolated IL-4RE (E5) or a trimeric construct (E1). The ISRE on its own (E8) had only a small effect, but in combination with the GAS element (E7) it stimulated a more than twofold upregulation of transcription. No constitutive repressor activities were detected, as none of the constructs had a lower transcriptional activity than the TK promoter alone.

The IL-4 antagonist [D124]IL-4 had a barely significant activity in the assay and [D121, D124]IL-4 had no effect at all with construct E2 (Fig. 4). IL-13 stimulated the E2 construct comparably to IL-4, while IFN-α and IFN-γ inhibited the IL-4-dependent increase of transcription, and by themselves downregulated transcription below the level found in the absence of any cytokine (Fig. 4). IL-4 could largely alleviate the suppression by IFN-α, but was much less efficient for counterregulating the effects of IFN-γ. Even a tenfold higher concentration of IL-4 was not sufficient to completely relieve the suppression induced by IFN-γ (data not shown).

To investigate the role of the central GAS element and its flanking regions for the regulation by IL-4, IL-13, IFN-α and IFN-γ, constructs E3, E6 and E7 were tested with these cytokines. Due to the possible binding of IFN-activated Stat factors to the potential ISRE site, construct E8 was studied as well (Fig. 4).

The isolated GAS element (E6) was sufficient for regulation by all four cytokines, but the responses were generally weaker than for construct E2. If either the IL-4RE (E3), or the ISRE (E7) were combined with the GAS element, IL-4 and IL-13 and were equally or sligthly more efficient than with the E2 construct, but both interferons were no longer able to mediate repression (Fig. 4). The potential ISRE alone (E8) showed a weak positive effect with IL-4 and a weak repression by IFN-α.

The concentration dependence for the stimulating effect of IL-4 was tested with construct E2. IL-4 upregulated reporter gene expression with an EC^{50} of 250 pM (Fig. 5A). The EC^{50} for IL-4-induced reporter gene activation did not correspond to the EC^{50} for IL-4-stimulated proliferation (Fig. 5A) or tyrosine phosphorylation of IL-4Rα (Fig. 5A, B), which were both 15 pM.

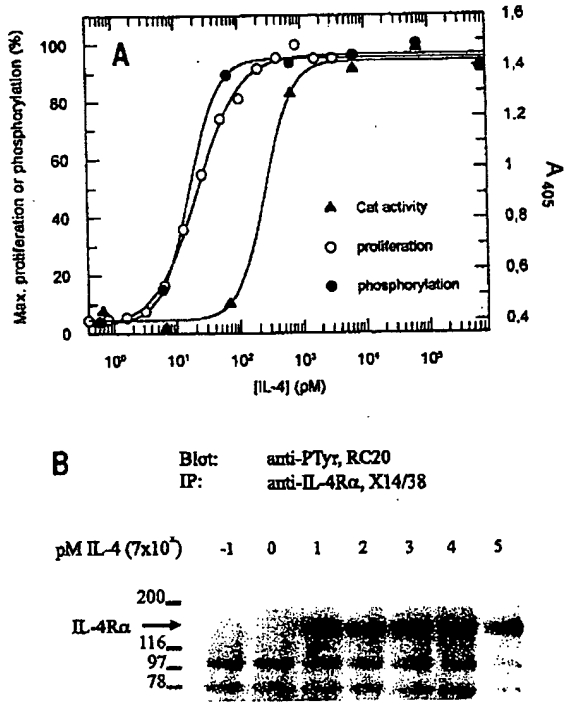

Fig. 5. Concentration dependence of effects of IL-4. (A) IL-4-induced cell proliferation (○), tyrosine phosphorylation of IL-4Rα (●) and CAT expression regulated by the E2 reporter gene construct (▲), in relation to IL-4 concentration. The EC^{50} for proliferation was 15 pM, the EC^{50} for reporter gene activity was 250 pM. (B) Tyrosine phosphorylation of IL-4Rα at various IL-4 concentrations. The EC^{50} of this response was 15 pM, as indicated in Fig. 5A.

DISCUSSION

The IL-4 receptor is a heterodimer of two members of the cytokine receptor superfamily, IL-4Rα and γc. However, some cell types lacking γc respond to IL-4, and others are not affected by inhibitory anti-γc antibodies (for review see [32]). Apparently these cells have a second type of IL-4 receptor, consisting of IL-4Rα and at least one unidentified subunit. It was shown by cross-linking that γc is a part of the IL-4 receptor complex in TF-1 cells [33], but considering the low level of γc transcripts found in northern blots, alternative forms of the IL-4 receptor could also be present. Because γc-knockout mice are unable to produce IgE [34, 35], B-cells seem to use the IL-4Rα/γc type of IL-4 receptor to signal Cε induction.

Previously, ε germline transcription has been investigated in various types of B-cells [9, 14, 15], and in the monocytic cell lines U937 and THP-1 [10, 11]. We have used the erythroleukemia line TF-1, because it responds to many cytokines, including IL-4 and IL-13 [28, 31]. The factors required for induction of germline transcripts seem to be rather widely expressed outside of the B lineage. All constructs tested showed an enhanced, constitutive expression in the absence of cytokines. Most efficient was construct E2, where transcription was tenfold higher than with the original vector pBLCAT5. This basal activity may be due to an enhancer activity of the inserted DNA fragments, or to the presence of constitutively active transcription factors in these cells.

There is a potential SP1 site in the human Cε promoter just downstream of the region investigated here [9]. IL-4-inducible nucleoprotein complexes similar to those which bind to an IL-4RE at the MHC A_α locus bind at the initiation site of ε transcription in the mouse [8], and upstream of the region investigated here [7]. In addition, the transcription factor BSAP (B-cell-specific activator protein), which binds to several other sites in heavy-chain switch regions [1], binds just upstream of the first initiation site for ε transcripts in the mouse [8, 20]. A region containing the BSAP binding site is sufficient for induction of transcription by CD40, but ineffective for stimulation by IL-4 [36]. All these sequences were lacking in our constructs and are therefore not strictly required for IL-4-induced transcription.

IL-4 induces binding of a Stat factor to another GAS site of the IgE germline promoter in the mouse [13], but this element is about 120 bp downstream of the GAS site which is critical for IL-4-mediated activation of germline transcription. In the human system, protein binding to the upstream GAS is induced by IL-4 [10, 11] and by IL-13 [37]. The element has the consensus sequence TTN_6AA for binding of IL-4 induced Stat complexes, which contain Stat6 [18, 38]. Transgenic mice lacking a functional Stat6 gene are unable to produce IgE [39–41].

Stepwise truncations have revealed that a sequence containing GAS is required for IL-4-induced transcription in the human Cε promoter, and has suppressor activity in the absence of IL-4 [14]. Both functions are destroyed by a single mutation within the inverted GAA repeat known to be essential for Stat binding [14, 17, 18]. GAS is therefore necessary, but a role for downstream sequences cannot be discounted.

We found that the GAS element alone was sufficient for IL-4-induced upregulation, but the best responses were obtained with a fragment that contained both upstream and downstream sequences. None of our constructs had factor-independent repressor activity. The repressor function of GAS which was identified by Albrecht et al. [14] seems to require interactions with sequences downstream of −61, which were not present in any of our constructs. The importance of the GAS box was underscored by a construct with three copies of GAS, which showed particularly increased transcription in the presence of IL-4. Similar results were reported using a reporter gene construct containing four copies of the human Cε GAS element in the cervical carcinoma line ME-180 [38].

In intact cells, the Stat factors binding to GAS may interact both with C/EBP proteins and NF-κB/p50, which bind within the Cε promoter close to the GAS site [15, 42]. Factors from the NF-κB and C/EBP families can productively interact to regulate gene transcription [43], and it has recently been shown that mice lacking a functional NF-κB/p50 gene are severly impaired in their synthesis of sterile ε transcripts and IgE antibodies [44]. Delphin and Stavnezer have suggested that the IL-4-responsive region of the murine Cε promoter binds simultaneously a Stat6-containing complex, one or more C/EBP factors and NF-κB/p50, and that the different factors interact with each other to regulate transcription [42]. The results presented here support a similar model for the human Cε promoter. In experiments with isolated elements from the murine Cε promoter, both the C/EBP-binding site and the GAS sequence were required for responsiveness to IL-4 [42]. In our experimental system, and in studies with truncated murine Cε promoter constructs by Rothman et al. [8], C/EBP was not required for regulation by IL-4. These discrepancies may be due to the different cell types used.

IFN-γ represses IL-4-induced germline transcription in a reporter gene assay with a 179-bp fragment of the IgE germline promoter in mouse B cells [12]. We have located the effect of IFN-γ to a 51-bp fragment which contains the GAS site. The same sequence was sufficient to mediate repression by IFN-α, which was found here to inhibit IL-4-induced transcription from the Cε promoter fragment. Both factors suppressed transcription in the absence of IL-4 to a comparable extent, but IL-4 was more efficient in alleviating the effects of IFN-α, which indicates that IFN-γ and IFN-α are not interchangeable for the regulation of IgE class switching. Both interferons can also suppress tran-

scription via the GAS element without its flanking regions, so this GAS seems to be a minimal responsive element for IFN-α and IFN-γ in the Iε promoter.

Repression by IFN-α and IFN-γ, but not upregulation by IL-4 and IL-13, is eliminated in constructs where only one of the flanking binding sites has been deleted, so interferon-stimulated Stat factors seem to be more dependent on interaction with other transcription factors at this site than IL-4/IL-13 induced Stat6 complexes. Stat complexes induced by IL-4 and IFN-γ are usually defined by different spacer lengths between the inverted GAA repeat [18, 38], but it has also been observed that Stat complexes activated by IL-4 or IFN-γ compete with each other for binding to shared response elements in the promoters for human CD23b and CD64 [45]. These complexes migrate on electrophoretic mobility-shift assays with different mobilities, and their exact composition is not known [45]. Competition between different transcription-factor complexes binding to the same DNA site is also likely to be involved in Cε promoter regulation.

Both the potential ISRE and the IL-4RE enhanced factor-independent transcription in our experiments, similar to results from another study [14]. The potential ISRE conferred a small positive response to IL-4, but IL-4RE alone was, despite its name, ineffective. This contradicts a previous report [9], a result which may be due to different cellular backgrounds. It is not clear how essential the human IL-4RE region outside of the C/EBP binding site is for regulation of germline transcription, because the similarity to the corresponding region in the mouse is relatively low [9].

Antagonistic mutant proteins of IL-4 fail to stimulate cellular responses, including T-cell proliferation and upregulation of CD23 by human B-cells [21–23]. [D121, D124]IL-4, a complete IL-4 antagonist, failed to induce transcription form the E2 construct as expected. [D124]IL-4, which has a small residual activity in CD23 induction assays [22] was also a partial agonist for induction of E2 transcription.

Induction of Cε reporter gene transcription is a low-affinity reaction, compared with receptor phosphorylation and proliferation in TF-1 cells. Several signaling pathways originate from the IL-4–receptor complex [32], which can by this means stimulate different sets of reactions depending on the IL-4 concentration present. Reporter gene systems like the one used here can help to unravel these complex cellular reactions.

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